Eur J Cancer Clin Onol. Vol. 17, No. 12, pp. 1241–1248, 1981.
Printed in Great Britain.

0277-5379/81/121241-08$02.00/0
Pergamon Press Ltd.

Dynamics of Serum Alpha-Fetoprotein During Spontaneous Hepatocellular Carcinoma Development in Mice

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Abstract—C3H–A^{vy}fB *mice have been studied over their normal life span and* 69% *developed hepatocellular carcinoma (HCC). Forty-seven mice were bled at monthly intervals, and individual serum alpha-fetoprotein (AFP) profiles are presented for* 36 *animals. Most of these profiles are quite irregular. Sixty percent of the* 15 *male mice had a transient serum AFP elevation at* 5 *months, reaching a mean of* 289 *ng/ml, compared to a mean of* 208 ng/ml *for those male mice without a transient AFP elevation and a mean of* 185 ng/ml *in male mice at 2–4 months of age. All of these male mice and others were tested for murine hepatitis virus and found to be negative. Mice with this early AFP peak tended to have a lower incidence of HCC and much lower AFP values later in life than those not having an early peak, but this difference was not statistically significant. Of the mice whose serum AFP concentration exceeded* 300 ng/ml *beyond* 6 *months of age,* 23% (9/39) *experienced a subsequent pronounced decline in AFP, averaging* 74% *of previous peak values for males and* 44% *for females. Of the* 39 *mice evaluated,* 35 *were autopsied and* 50% (4/8) *of these "serum AFP regressors" had microscopic evidence of lymphocytic infiltration in their livers, as compared to* 15% (4/27) *of the "non-regressors" that had a similar change.*

INTRODUCTION

SINCE the original discovery of alpha-fetoprotein (AFP) in mice bearing transplantable tumors [1], this tumor marker has been shown to be correlated with the growth of hepatocellular carcinoma (HCC) in other species as well as man, and has been used as an aid to diagnosis in selected populations of individuals [2, 3]. Many studies of AFP and HCC have utilized chemical carcinogens, and others have utilized spontaneous tumor models, usually the C3H mouse, which represents a strain with a variable, but usually high, incidence of HCC [4–9]. Becker *et al.* [6] reported the incidence of AFP and HCC in strain C3H–A^{vy}fB mice at different ages. Jalanko *et al.* [10] studied C3H/A/BOM and C3HeB/FeJ mice at 1–4 month intervals and showed a biphasic serum AFP elevation in some of the animals. The purpose of this study was to examine the dynamic changes in serum AFP during HCC development in C3H–A^{vy}fB mice [4] by frequent bleeding over the entire life span. A previous publication from this laboratory [11] reported results on a portion of the group of animals reported here. That report includes information on the correlation of serum AFP and gross liver disease, and the lack of correlation between serum AFP and degree of tumor differentiation, which is not included in the current report. Also, the current report includes a larger number of animals and more extensive analysis of the pathology and serum AFP data than was previously available.

Accepted 9 June 1981.
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MATERIALS AND METHODS

Mice

Ninety-three C3H–A^{vy}fB mice, 37 males and 56 females, aged between 2 and 23 months,

were studied. All were housed 4–6 per cage and fed standard lab chow and water *ad libitum*. A group of 46 mice was bled and serum was assayed at autopsy. The other mice, 22 males and 25 females, were bled at monthly intervals, and killed and autopsied when general signs of ill health became apparent. Serum AFP values, obtained at 2–8 months of age from 16 mice in the first group and from 28 serially-bled mice, were used as normal controls for both groups in later life, when many mice would be expected to develop liver pathology-associated serum AFP elevations. Seven males and five females from the serially-bled group were not available for autopsy. Each animal was bled from the retro-orbital venous sinus, and serums were stored at −20°C in plastic micro-centrifuge tubes until termination of the study. A detailed gross description of the liver was recorded and tissues were fixed in Tellyesniczky's fluid and stained with hematoxylin and eosin. Slides were reviewed for histology without knowledge of the AFP value.

Radioimmunoassay

Serum AFP was quantitated by a double-antibody, single-isotope radioimmunoassay (RIA). The method of Kortright *et al.* [12] was used with only slight modifications, which included elimination of the 2-hr pre-incubation with first antibody at 37°C, and elimination of normal serum in every tube. The assay utilized a rabbit anti-mouse fetal AFP antiserum which had been adsorbed with insolubilized adult normal mouse serum proteins in order to render it monospecific. Affinity chromatography, followed by polyacrylamide gel electrophoresis, was utilized in the purification of mouse fetal AFP, which was employed as both the standard and the radiolabelled antigen. Labelling of the purified AFP by the Chloramine-T method gave a specific activity of 91 μCi/μg. Ten consecutive assays had a mean slope of −0.943, with curve limits of 92.4–2.7% precipitation (22–11,200 ng/ml). The maximum binding averaged 38.4%, with nonspecific binding of 2.9%. The assay standards had a mean standard error of 0.17 ± 0.02 and the correlation coefficient averaged 0.996. The performance of quality control standards is listed in Table 1. The fetal mouse extract, which provided the immunogen, was kindly quantitated by Dr. E. Ruoslahti. That value was later confirmed against our own purified AFP and the same extract was used as displacer in the RIA. Serums were assayed on 50 μl duplicates.

RESULTS

Tumor incidence and pathology

Seventy-nine percent (64/81) of the mice autopsied had grossly visible liver nodules which were yellow-white to red-brown in color, and 1–12 mm in diameter. The 17 mice with grossly normal livers were all 6–9 months of age. Not all mice with gross nodules had HCC and 47% (8/17) of those that appeared normal had microscopic HCC. Table 2 lists the occurrence of liver nodules and incidence of HCC by sex. Microscopically, all livers were abnormal, with diffuse and/or nodular hyperplasia, or HCC. The HCC ranged from well to poorly differentiated and often contained large sinusoids, constricted segments and areas of fatty degeneration or lymphocytic infiltration. Nine percent (5/56) of the HCC had metastasized, 4 to the lung and 1 to both lung and kidneys. Thirty-five percent (28/81) of the mice autopsied had a total of 39 primary tumors other than HCC; 23 of those 28 mice also had primary HCC. The non-HCC tumors included: 21 mammary tumors, 11 lung tumors, 3

Table 1. *Performance of control serums in six consecutive radioimmunoassays for mouse alpha-fetoprotein (AFP)*

Sample	Mean serum AFP, ng/ml	S.D.	Coefficient of variation, %
Control-1	197.3	27.4	13.9
Serum pool	592.6	19.2	3.2
Control-2	1544.5	84.9	5.5
HCC serum	1,469,480	64,766	4.4

Table 2. *Gross pathology and tumor incidence*

	No. mice autopsied	Gross liver pathology		Microscopic liver pathology		HCC* months
		Nodules	Normal	HCC	(%)	
Male	30	21	9	17	(57)	14.3
Female	51	43	8	39	(76)	16.2
Total	81	64	17	56	(69)	

*Hepatocellular carcinoma: mean age at which HCC was observed at autopsy.

cholangiomas, 2 reticulum cell sarcomas, 1 fibrosarcoma of the kidney and 1 hemangioendothelioma. Turusov *et al.* [13] believe that the cholangiomas should be regarded as tumors resembling human hepatoblastoma. All three cholangiomas occurred in mice which also had HCC and elevated serum AFP. Some animals had two or even three primary tumors. The non-HCC tumors were found in mice that were 15–23 months of age except for one at 10 months, and occurred at a mean of 17.6 months, median 17.0 months.

Serum studies

The normal serum AFP concentration for 22 males at age 2–4 months was a mean of 185 ng/ml, and the mean plus 2 standard deviations (S.D.) was 264 ng/ml. For 22 females at age 2–8 months, the mean was 141 ng/ml, and the mean plus 2 S.D. was 209 ng/ml. There appeared to be a strong positive correlation between the extent of gross liver disease and the serum AFP concentration at the time of autopsy. Only 12% (2/17) of those animals with no gross liver disease had serum AFP > 300 ng/ml, while 88% (43/49) with multiple nodules or massive liver disease had serum AFP > 300 ng/ml (chi-square = 33.6, $P < 0.01$). Both mice with elevated serum AFP, but no gross liver disease, were later found to have microscopic evidence of HCC.

AFP distribution by pathology and sex

Figure 1 displays the distribution of peak serum AFP values, indicating that the range of these values is quite similar for both sexes. Those mice with only hyperplastic changes of the liver all had serum AFP values of 900 ng/ml or less. The mean values for the 13 males (244 ng/ml) and 12 females (336 ng/ml) with hyperplasia are at or slightly above the upper limit of normal, which is defined here as the mean concentration for normal animals plus 2 S.D. There were some tumor-bearing mice whose tumors did not secrete AFP, since 21% (12/56) of the mice with HCC had serum AFP values below the upper limit of normal. Males with HCC had a substantially higher mean AFP concentration than females with HCC, 58,808 ng/ml vs 11,471 ng/ml (median, 2,279 vs 653 ng/ml). This difference was examined using Student's *t*-test and found to be significant ($P < 0.01$). About 79% of the mice with HCC had serum AFP > 2 S.D. above the mean. The highest concentration was in a male, 439,040 ng/ml. Of the eight mice with microscopic HCC but no gross nodules, two had elevated AFP, 625 and 1,077 ng/ml. None of the nine mice with hyperplasia but lacking gross nodules had elevated serum AFP.

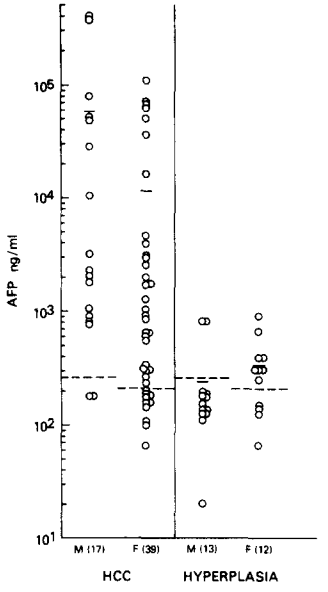

Fig. 1. The distribution of individual highest serum AFP values by liver nodule pathology and sex. Numbers in parentheses are numbers of mice in each category. Short dashed lines indicate normal serum AFP mean plus 2 S.D. Bars represent mean levels for each category.

Serial AFP data

Figures 2a–f present serial serum AFP profiles on 30 of the 47 mice bled monthly. Six others will be presented in a subsequent section and 11 others are not presented in detail here because the mice were not able to be followed for more than a few months, or the AFP levels remained below 300 ng/ml and were of less interest. The serial sampling shows that AFP levels remained normal in some mice throughout their lifetime, some rose above the upper limit of normal only late in life, while others rose above the normal range as early as 6–7 months of age. Most animals did not have a smoothly rising AFP course but had profiles punctuated by peaks, valleys and plateaus. This included some animals whose serum AFP remained below 300 ng/ml. The configuration of these curves varied considerably, both between different animals and for a given mouse at different times. Examples of this latter difference can be clearly seen in the profiles of male mice nos. 36, 795, 40 and 37, in Figs. 2a and b.

Sex differences

Among the sex differences was the disparity in HCC incidence. Males had a rate of 57% at a mean autopsy age of 14.3 months while females had a rate of 76% at a mean autopsy age of 16.2

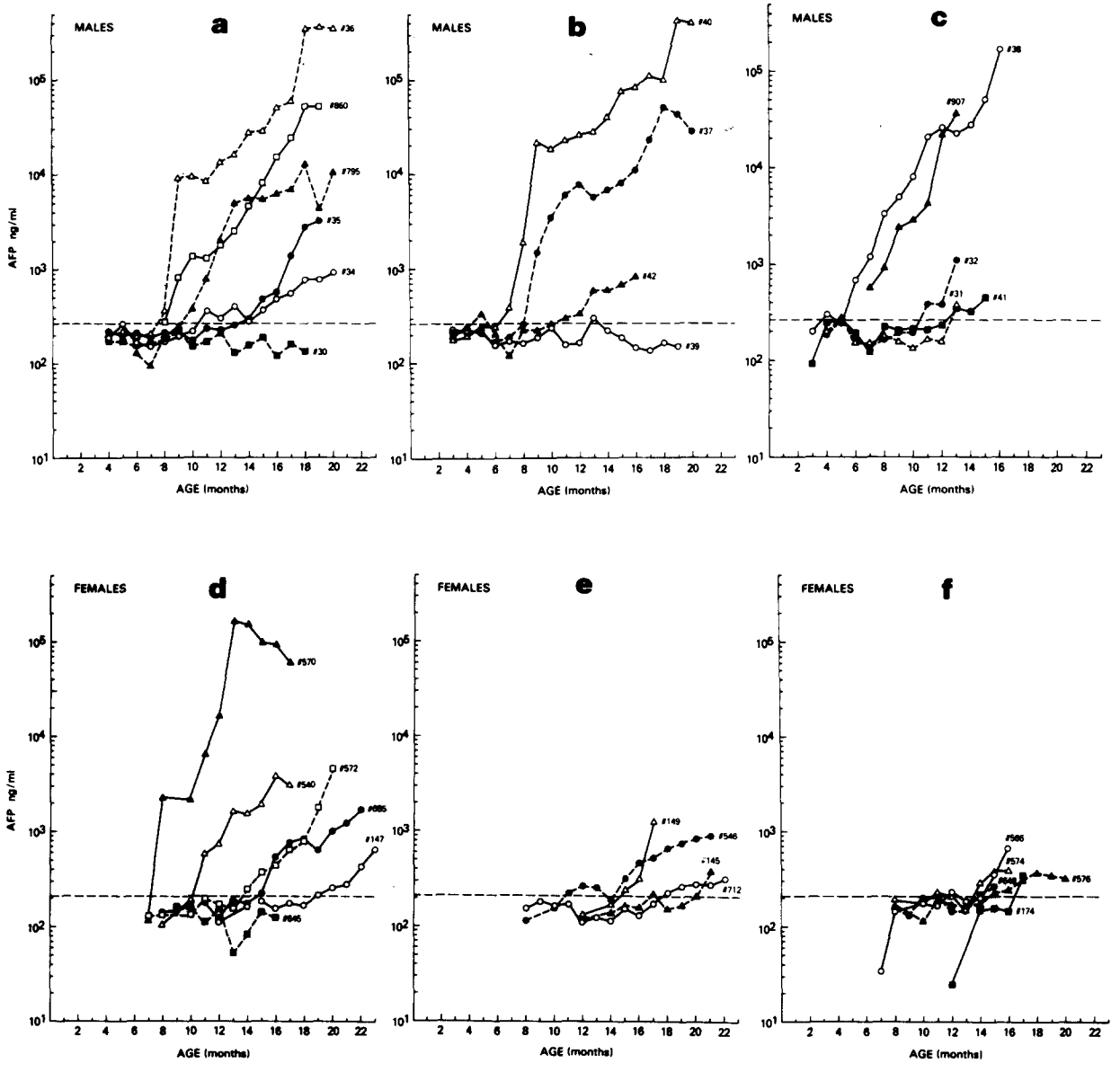

Fig. 2(a–f). Serial serum AFP profiles for 15 males and 15 females. Dashed lines represent the normal serum AFP mean plus 2 S.D., 264 ng/ml for males and 209 ng/ml for females. Panels a–c contain male profiles grouped for spacing effect only. Panels d–f contain female profiles, with those reaching the highest serum AFP levels grouped in panel d.

months, but this difference was not significant. However, males had a higher rate of AFP-positive HCC, 88 vs 74%. The mean serum AFP concentration was 58,808 ng/ml for all males with HCC and 11,471 ng/ml for females with HCC ($P < 0.01$). More males also reached serum levels of 300 ng/ml or greater (77 vs 64%) and did so much earlier, having a mean of 8.6 months versus a mean of 15.7 months for females. This age difference was tested using the Mann–Whitney test and found to be significant ($P < 0.01$). The difference is quite obvious when Figs. 2a–c are compared with Figs. 2d–f. Also, a greater proportion of the females failed to exceed 900 ng/ml.

Early AFP rises

In the serially bled mice, 15/22 males had blood drawn at 4–6 months of age. Sixty percent (9/15) showed a serum AFP peak at or near 5 months of age, followed by a decline, usually to the normal range. The mean for this group was 289 ng/ml at 5 months, compared to a mean of 208 ng/ml for those of the same age which did not show this peak. This difference was examined using Student's t test and found to be significant ($P < 0.01$). The mean for 2–4 month-old males was 185 ng/ml. The range of peak serum AFP levels for those nine mice was 248–339 ng/ml, compared to a range of 169–226 for those not showing a peak and 92–255 for

normal young males at 2–4 months of age. Figure 3a presents profiles of those nine mice at this early period. Only mouse no. 38 deviates from the very consistent peak at 5 months by peaking at 4 months. The profiles for nos. 38 and 43 did not return to normal, but after the partial decline shown, rose again and quickly progressed to high levels of AFP (Figs. 2c and 4a). Figure 3b presents profiles for the six male mice which did not show a peak at 5 months of age. Sera of these 15 male mice were all measured in the same assay during the period of change from baseline to elevations at 5 months, including those which did not show the increase. All male mice in the serially-bled group were tested for murine hepatitis virus at 5 months of age and found to be negative.

There were other differences between the groups with and without the peak at 5 months. The subsequent highest AFP levels achieved were median values of 820 ng/ml for those with an early peak at 5 months and 15,668 ng/ml for those without the early peak. This difference in serum AFP levels was tested using Student's *t* test and found to be significant ($P < 0.01$). Autopsies were not feasible on five of the nine mice in the early peak group. However, only 25% (1/4) of the mice in that group developed HCC vs 67% (4/6) in the group without an early peak. Due to the small number of observations, chi-square testing yields a value of only 1.7, P = approximately 0.19. No females were serially bled between 4 and 6 months of age.

Serum AFP "regressors"

In the group followed serially, 25% (5/20) of the males and 21% (4/19) of the females with at least 5 consecutive monthly serum samples between 10 and 19 months of age had AFP profiles showing a marked decline. This was defined as two or more successive monthly AFP values lower than a preceding point which had reached at least 300 ng/ml, and consisted of at least a 30% decline in serum AFP concentration. In five of these nine "regressors", the serum AFP decline included values which dropped progressively over a period of 2–4 months. These "regressions" were observed over the period of 10–19 months, with a mean age at occurrence of 14.3 months for the five males and 15.9 months for the four females. For the five males, declines of 43, 67, 76, 90 and 96% of the preceding peak AFP concentration were seen. Females registered drops of 31, 32, 48 and 63%. The mean for males was 74% and for females was 44%. This difference was examined using Student's *t*-test and found to be significant ($P < 0.01$).

Figure 4 shows the profiles of three males and four females with such declines. Both males no. 856 and no. 953 had varied AFP courses which included a sharp decline and also plateaus, which interrupted a steep rise. The liver tumor of no. 856 was characterized as having areas of lymphocytic infiltration. The profile for male no. 43 showed a steady climb to more than 20,000 ng/ml AFP, followed by a 96% decline

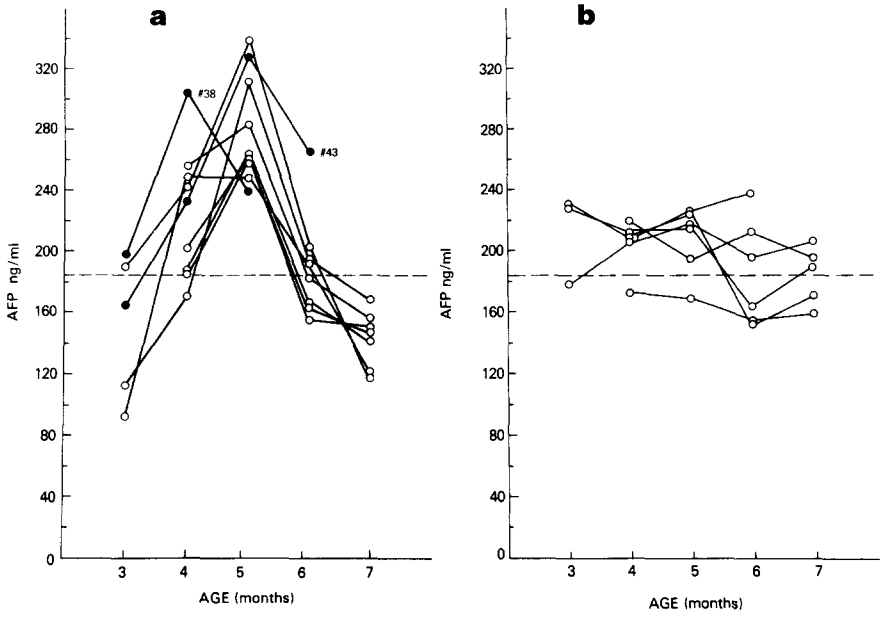

Fig. 3(a and b). Comparison of serum AFP profiles of 9 male mice with early, transient AFP peak at 5 months of age (panel a) and 6 male mice without a peak at 5 months (panel b). Values for mice no. 38 and no. 43 (solid circles, panel a) did not return to previous lower levels, as did the others. Dashed lines represent mean for normal males at 2–4 months of age.

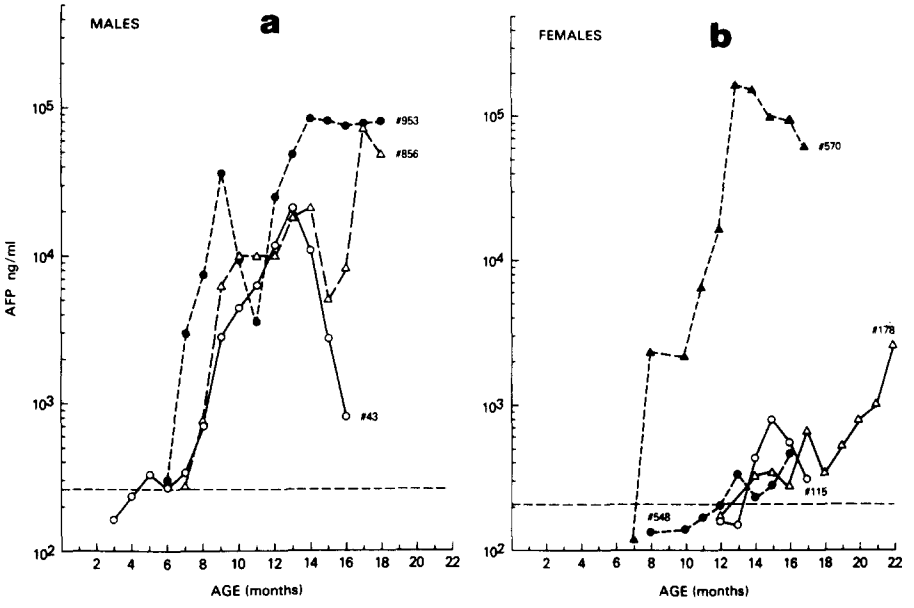

Fig. 4(a and b). Serial serum AFP profiles of 3 males (panel a) and 4 females (panel b) which showed spontaneous "serum AFP regression." Dashed lines represent normal serum AFP mean plus 2 S.D. Substantial declines in the serum AFP levels occurred at different ages and sometimes were followed by recovery to higher AFP levels.

over a 3-month period. At autopsy, the AFP concentration was only 805 ng/ml and the liver was characterized, without knowledge of the serum data, as having only hyperplasia, but no lymphocytic infiltration. Female no. 570 had an early interruption in the AFP rise at 8–10 months, and after a subsequent sharp rise to 168,910 ng/ml, experienced a 63% decline over the next 4 months and showed only hyperplasia and lymphocytic infiltration in the liver. Female no. 115 only rose to 789 ng/ml but dropped to 313 ng/ml, and was characterized as having lymphocytic infiltration, extensive necrosis and hyperplasia without HCC. Females no. 178 and no. 548 had much less dramatic declines but did fit the "regressor" criteria. Mouse no. 178 had HCC without lymphocytic infiltration and no. 548 was not autopsied.

The other two mice with peaks >300 ng/ml and classified as "serum AFP regressors" were males no. 795 and no. 37, shown in Figs. 2a and 2b respectively. There was lymphocytic infiltration in the liver of no. 37. Eight of the nine "regressors" were autopsied and five of those eight were judged to have HCC at autopsy. The other three (nos. 856, 570 and 115) had only hyperplasia but had high serum levels of AFP 2–4 months earlier.

Fifty percent (4/8) of the autopsied "regressors" had lymphocytic infiltration in the liver. Only 15% (4/27) of the serially-bled "non-regressors" had lymphocytic infiltration. Chi-square testing of these groups yields a value of 4.3, indicating a significant difference, $P = 0.04$. In contrast, analysis of the occurrence of lymphocytic infiltration of the liver when mice were grouped by serum AFP level does not show a correlation with this parameter. Of the mice with "low" serum AFP (<300 ng/ml) 6% (2/31) had lymphocytic infiltration, while 20% (10/50) of those with "high" serum AFP (>300 ng/ml) had such infiltration. Testing of this difference gave values of chi-square = 2.78, $P = 0.10$.

DISCUSSION

The incidence of HCC and the association of HCC and AFP in the present study were not substantially different from previous reports on this and related strains [4–8]. The finding that males tended to produce greater concentrations of AFP and did so earlier than females is not new [6] and, although unexplained, will receive no further attention here.

The primary reason for doing this study was to follow the AFP course of individual animals. Therefore, we have presented profiles on 36 mice, 18 of each sex. One of the more interesting findings was an early transient AFP elevation in a majority of male mice at 5 months of age. No female mice were serially bled between 4 and 6 months of age. Similar findings have been made by others in studies on mice [10]. Early, transient serum AFP elevations

have also been found in studies of chemical carcinogenesis in rats [14, 15]. Previously unreported, however, were the observations that: (1) those mice with the early serum AFP peak had peak levels later in life that were only about 5% of that in mice that did not have an early peak; and (2) that substantially fewer mice in that early peak group developed HCC than those with no early peak. A possible explanation for these results is that the early AFP peak reflected cellular changes which sensitized the host and generated an immune response, which suppressed subsequent HCC development and therefore also serum AFP levels. This early AFP rise was apparently not due to infection with hepatitis virus. However, the number of observations here is small, especially for the comparison of pathologic findings, and these differences cannot yet be called significant.

It is clear that there is much variation in the rate and degree of AFP progression and that very few mice had steady, rising profiles. The situation may be made more complex due to varying numbers of newly arising foci of malignant cells at different times and with different growth rates. The extensive monitoring demonstrated the occurrence of "serum AFP regressions." This included 23% of the mice which were sampled on at least five consecutive months between 10 and 19 months of age and exceeded 300 ng/ml. These "regressions" were followed by subsequent elevations in most mice after 2 months, but in a few the decline was sustained for several months without a subsequent elevation during the follow-up period.

Of the three "regressor" mice with no histologic evidence of HCC but only liver hyperplasia at autopsy, it is possible that mouse no. 115 never had HCC, since the peak AFP value of 789 ng/ml could have been due to hyperplasia alone. Mouse no. 570 would have been expected to have HCC with an AFP level of 62,260 ng/ml even after a 63% decline over 4 months, and may have had HCC that was not detected. Mouse no. 43 may actually represent an HCC that was rejected, since the peak level of 21,380 ng/ml was much higher than would have been expected in association with hyperplasia alone. It is interesting to speculate that these changes in serum AFP concentration may reflect diminution of tumor due to host response, especially since spontaneous regression of HCC in other species is apparently a rare event. A more detailed study of these events in the C3H–A^{vy}fB model, with attention to immune functions, might be helpful as better methods are sought to deal with HCC in humans.

Acknowledgements—We thank the staff of the Animal Health Diagnostic Section, Frederick Cancer Research Center, Frederick, Maryland, for testing sera for the presence of murine hepatitis virus. We are also grateful to Dr. Ronald Herberman for reviewing the manuscript and making valuable comments.

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